Original Contribution

Circulation Cooling in Continuous Skin Sonoporation at Constant Coupling Fluid Temperatures

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Abstract

Exposure of the skin to low-frequency ultrasound in the Franz diffusion cell has been found to increase the permeability of the skin to molecular transport. In many cases, significant heating of the coupling fluid requires the use of duty cycles that extend the total experimental time. This is a methodological study in which the coupling fluid is circulated between a modified Franz diffusion cell and a heat exchanger to allow for the continuous application of low-frequency ultrasound while the coupling fluid temperature is held constant. Dermatomed porcine skin was exposed to continuous ultrasound at 20 kHz for 10 min at an intensity of 55 W/cm^2 while the coupling fluid was maintained at one of three target temperatures (13°C, 33°C or 46°C). Foil pitting and passive cavitation detection revealed that inertial cavitation activity decreased with increasing coupling fluid target temperature. Transport measurements revealed an increase in mean donor calcein concentration with increasing coupling fluid temperature, though these were not statistically significant. Taken together these findings suggest that the weakened stratum corneum lipid structure at higher temperatures is more susceptible to the introduction of defects from the jetting of cavitation.

Key Words: Skin permeability; Sonoporation; Transdermal drug delivery; Ultrasound; Sonication; Temperature; Franz

Introduction

Sonoporation, also referred to as pre-treatment sonophoresis (Mitragotri and Kost 2004), involves exposure of the skin to ultrasound (before application of the drug) to increase the skin's permeability. Under certain conditions, cavitation nuclei in the coupling fluid (the medium between the transducer and skin) grow to form cavitation bubbles, which then violently collapse upon compression (Mitragotri and Kost 2004; Helga et al. 2015). This violent inertial cavitation activity near the surface of the skin results in microscopic defects in the lamellar lipid structure of the stratum corneum (SC) (Ueda et al. 1996, 2009; Wu et al. 1998; Mitragotri and Kost 2004; Smith 2007; Dahlan et al. 2009), and this is believed to be the mechanism underlying the observed increases in the skin's permeability to drugs (Mitragotri et al. 1995, 2003; Tezel et al. 2001, 2002; Tang et al. 2002; Alvarez-Román et al. 2003; Tezel and Mitragotri 2003; Lee et al. 2010; Polat et al. 2010).

In vitro skin sonoporation experiments have traditionally been conducted in Franz diffusion cells (Mitragotri et al. 2000a; Tang et al. 2002; Terahara et al. 2002; Lavon et al. 2005; Paliwal et al. 2006; Herwadkar et al. 2012; Han and Das 2013). A transducer is inserted into the donor chamber and emits an ultrasound field that propagates through the coupling fluid toward the skin. When the ultrasound waves travel through the coupling fluid, a fraction of the mechanical energy is converted to thermal energy (O'Brien 2007). This absorption heating can result in a significant increase in the coupling fluid temperature (Tang et al. 2002; Terahara et al. 2002), which must be controlled as the skin may be permanently damaged at prolonged exposure to temperatures >43°C (Moritz and Henriques 1947; Lindeque et al. 2013; Hao et al. 2016).

Traditionally (in passive diffusion studies), only the temperature of the receiver chamber has been regulated using a water jacket to simulate *in vivo* conditions. One study (Addicks et al. 1987) modified a flow-through receiver chamber to control the temperature of the donor chamber. But such apparatus are not equipped to remove heat at the rates experienced in skin sonoporation experiments. To avoid excessive heating of the coupling fluid, researchers have used either duty cycles, coupling fluid replacement or a combination of the two (Tezel et al. 2001, 2004; Tang et al. 2002; Terahara et al. 2002; Lavon et al. 2005, Paliwal et al. 2006; Polat et al. 2011).

There are still opportunities to improve the experiment. Duty cycles may significantly increase the total time required to conduct a set of experiments (compared with continuous ultrasound application); as an example, in the study by Tang et al. (2002), the use of a 10% duty cycle resulted in a total experimental time 100 h longer than would have been required for continuous ultrasound. The method of fluid replacement can require much more intervention; in the study by Tezel et al. (2004), the coupling fluid was replaced four times in each experiment. Although duty cycles and fluid replacement prevent the coupling fluid from reaching temperatures that could permanently damage the skin, their use has been reported to result in temperature fluctuations of up to 20°C (Tang et al. 2002; Terahara et al. 2002) and to result in rates of increase of 1°C/min Lavon et al. (2005). Because significant changes in the skin's permeability at even moderate changes in temperature (in the range 20–45°C) have been reported to be a result of SC lipid melting (Chang and Riviere 1991; Clarys et al. 1998; Ogiso et al. 1998; Shahzad et al. 2015), it follows that the coupling fluid temperature may influence the permeability changes of the SC.

In this article, we describe a methodological study that was motivated by the fluctuating coupling fluid temperatures of existing experiments reported. We first wanted to modify the Franz diffusion cell so that continuous ultrasound could be applied to the skin to avoid the additional time associated with the duty cycle. Additionally, we wanted to be able to keep the coupling fluid temperature constant during sonoporation to observe the influence of coupling fluid temperature on the cavitation and subsequent permeability increases.

Methods

Modified diffusion cell

The modified Franz diffusion cells developed for this study are similar to those used by Robertson and Becker (2018). The diffusion cell was designed (Fig. 1a) so that during ultrasound application, the coupling fluid could be circulated from the donor chamber to a heat exchanger. The donor chamber had an outer diameter of 65 mm, an inner diameter of 61 mm, an aperture diameter of 9 mm and a total volume of 96 mL. Each receiver chamber had a volume of 3.2 mL and an aperture diameter of 9 mm.

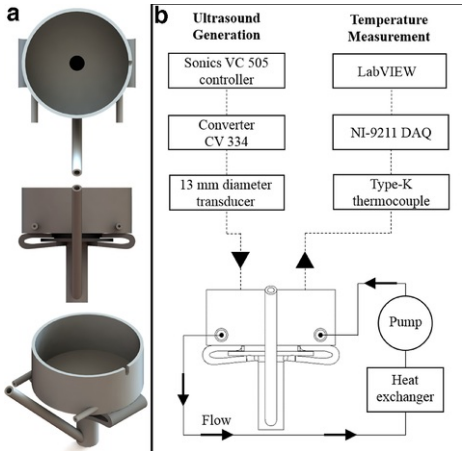

Fig. 1 (a) The modified diffusion cell (top) plane view, (middle) front view and (bottom) isometric view. (b) System schematic. Solid lines represent the coupling fluid circuit. Dashed lines represent the signal inputs to the transducer and the signal outputs from the thermocouple.

alt-text: Fig 1

The donor and receiver chambers were turned from solid polypropylene rods (Polystone, Dotmar EPP Pty Ltd., Christchurch, New Zealand) on a computer numerical control lathe (Top-Turn CNC 406, Jashco Machine Manufacture Co. Ltd., Taichung, Taiwan). The receiver chamber sampling arms and donor chamber ports were then retrofitted by gluing (Loctite 401, Henkel AG, Düsseldorf, Germany) sections of carbon fiber tube (Carbon Fibre Tube Pultruded, MAKERshop, Auckland, New Zealand) into holes that were drilled into the polypropylene. The receiver sampling arms had an inner diameter of 3.5 mm and an outer diameter of 6 mm. The donor ports had an inner diameter of 2 mm and an outer diameter of 4 mm. The clamps that held the donor and receiver chambers together were 3-D printed from acrylonitrile butadiene styrene (ABS P430, Stratasys, Eden Prairie, MN, USA).

Chemicals

Calcein has been used in previous transdermal transport studies (Henry et al. 1998; Prausnitz et al. 1993; Vanbever and Preat 1999; Xie et al. 2005; Oh et al. 2008). In this study a permeant solution was used that consisted of calcein (Calcein C0875, Sigma-Aldrich, St. Louis, MO, USA) dissolved in phosphate-buffered saline (PBS) (Gibco PBS, pH 7.4, Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 0.1% w/v (1 g/L). This solution was made and

stored at room temperature.

Porcine skin

The *in vitro* skin models of this study consisted of porcine ear skin. Porcine skin has been used in sonoporation and sonophoresis experiments (Mitragotri et al. 2000a; Tezel et al. 2001, 2002; Tang et al. 2002; Terahara et al. 2002; Tezel and Mitragotri 2003; Alvarez-Román et al. 2003; Paliwal et al. 2006; Sarheed and Abdul Rasool 2011; Polat et al. 2012; Schoellhammer et al. 2012; Souza et al. 2013; Lee and Zhou 2015; Zorec et al. 2015). Porcine ears were obtained from an abattoir (Ashburton Meat Processors Ltd, Ashburton, New Zealand) immediately after slaughter. These ears were not scalded or exposed to detergent at the abattoir. Upon arrival at the laboratory, the ears were cleaned with cold tap water to remove the blood and soil. The skin was then removed from the ears using a dermatome (Dermatome 50 mm, Nouvag AG, Goldach, Switzerland) set to a thickness of 1 mm. The dermatomed skin samples were wrapped in Parafilm (PARAFILM, Sigma-Aldrich), placed in sealed plastic containers and then transferred to a –20°C freezer until use. The total time from slaughter to freezer was less than 4 h, and skin samples were used within 18 d.

Ultrasound generation and intensity measurement

The continuous low-frequency (20-kHz) ultrasound field was generated with an ultrasonic processor (VC 505 Ultrasonic Processor, Sonics and Materials Inc., Newtown, CT, USA). The distance between the transducer and the target (skin or foil) was 3 mm. The intensity of the ultrasound field was determined using the calorimetric method (Mitragotri et al. 2000a, 2000b; Terahara et al. 2002; Alvarez-Román et al. 2003; Mutoh et al. 2003; Morimoto et al. 2005). The ultrasound transducer was positioned in a beaker of water embedded in a thermally insulating block of polystyrene, and the temperature was taken using a wire type K thermocouple (Jaycar Electronics Pty. Ltd., Auckland, New Zealand). The ultrasound intensity was calculated as

$$I = \frac{mC_p}{A}\frac{\Delta T}{\Delta t} \quad \textbf{(1)}$$

where m is the mass of water in the beaker (0.2 kg), C_p is the specific heat of water (4.187 kJ/kg K), A is the active transducer face area (1.3 cm^2) and ΔT is the measured change in temperature that occurred during the 3 min (Δt) of ultrasound exposure. Each of these intensity measurements was run in triplicate.

Temperature measurement

The temperatures were taken with wire type K thermocouples (Jaycar Electronics Pty. Ltd., Auckland, New Zealand) at a sampling frequency of 1 Hz. The readings from the thermocouple were passed to the NI-9211 DAQ module (National Instruments Ltd., Austin, TX, USA) then on to a PC for processing in LabVIEW (National Instruments Ltd.).

Measurement and regulation of coupling fluid temperature

The temperature control system of this study was based on that used in the study by Robertson and Becker (2018). The coupling fluid (de-ionized water) was circulated out of the donor chamber through an outlet port, through a heat exchanger and back into the donor chamber through an inlet port (as shown in Fig. 1b). A peristaltic pump (6-V Peristaltic Pump, Amazon, Seattle, WA, USA), driven by a power supply (MP3090, Powertech, Taiwan), maintained a constant flow rate of 0.14 L/min around this circuit. The heat exchanger consisted of channels that were milled into an aluminum block that was submerged in a temperature-controlled water bath (FP-50 Refrigerated/Heating Circulator, Julabo GmbH, Selbach, Germany). When the coupling fluid passed through channels milled within this block, the coupling fluid temperature tended toward the temperature of the water bath.

The temperature of the coupling fluid during ultrasound application was set by adjusting the water bath temperature. The components were linked by silicone tubing (Silicone Tubing Clear Grade Inner Diameter 2.6 mm, Rubbermark Industries, Auckland, New Zealand) that could easily be removed from the donor chamber ports.

The temperature of the coupling fluid was measured during each transport experiment. The location of the thermocouple was 8 mm above the bottom donor chamber wall and 7 mm from the transducer (qualitatively depicted in Fig. 2a). This position was chosen to ensure that the thermocouple was outside the transducer beam during ultrasound application—the thermocouple tip was above the level of the donward-facing transducer tip and, therefore, outside of the beam. We positioned the thermocouple this way because we did not not want it to interfere with the ultrasound field or with the resulting local fluid dynamics near the skin during the transport experiments.

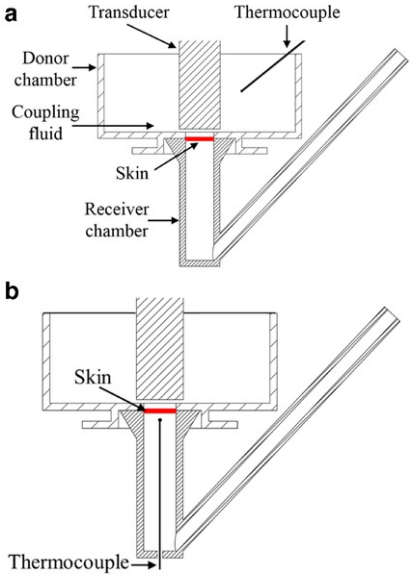

Fig. 2 (a) Cross-section of the modified diffusion cell revealing the positioning of the transducer and thermocouple during sonoporation. The thermocouple was 8 mm above the bottom donor chamber wall and 7 mm from the transducer edge. The hydrophone tip was positioned 5 mm above the transducer face and 7 mm from the skin aperture. (b) Cross-section of the sacrificial diffusion cell revealing the positioning of the thermocouple used to measure the receiver fluid temperature. The thermocouple tip was positioned 2 mm below the bottom surface of the skin.

alt-text: Fig 2

Although we did not measure the skin temperatures during the transport experiments, we did conduct a separate (from transport experiments) thermal analysis of the temperature of the skin exposed to continuous ultrasound with circulation for which a second thermocouple was positioned directly onto the skin and inside the transducer beam. These experiments were conducted at each of the three target coupling fluid temperatures with ultrasound at an intensity of 55 W/cm^2.

Measurement of the receiver fluid temperature

To measure the heat transfer to or from the receiver fluid, a hole was drilled in the bottom of a sacrificial receiver chamber (this was not used in the transport experiments). A type K thermocouple was then inserted through this hole so that its tip was positioned 2 mm below the skin surface (Fig. 2b). Glue (Loctite 401, Henkel AG) was used to secure the thermocouple wire to the receiver chamber to prevent any leakage and to ensure that the thermocouple remained in position. The thermocouple recorded data for a total of 30 min. The first 10 min corresponded to the time of ultrasound application, or the time that water was circulated through the donor chamber in the cases without ultrasound. The receiver fluid temperatures were recorded for an additional 20 min; this period would correspond to the first 20 min that the permeant was applied to the skin.

Measurement of the permeant solution temperature

After the 10 min of coupling fluid circulation (with or without ultrasound), the coupling fluid was removed from the donor chamber. The calcein-buffer solution was then added to the donor chamber so that diffusion could begin. During the first 15 min of this diffusion, the temperature of the calcein-buffer solution was measured with a type K thermocouple which was positioned inside the buffer solution 1 mm above the skin surface.

Foil pitting experiments

The pitting of aluminum foil has been used to quantify the influence of different experimental parameters on the inertial cavitation activity (Mitragotri et al. 2000a). This technique was used to investigate the influence of coupling fluid temperature on the inertial cavitation activity. Pieces of aluminum foil were placed in the modified diffusion cells and exposed to 1 s of ultrasound at an intensity of 55 W/cm^2 with the coupling fluid at 10°C or 40°C ($n = 10$). Foil pitting experiments were also used to investigate whether there was any interference of the circulation of the coupling fluid on the inertial cavitation activity. The aluminum foil samples were exposed to ultrasound at an intensity of 55 W/cm^2 for 2 s with circulation or without circulation ($n = 10$) of the coupling fluid at 10 ± 1°C.

Passive cavitation detection

Inertial cavitation activity has also been reported to be represented by broadband noise which occurs between the subharmonic, harmonic and ultraharmonic peaks (Yao-Sheng et al. 2010). An additional set of experiments (n = 7) were conducted to monitor the inertial cavitation activity during sonoporation in which ultrasound was applied at an intensity of 36 W/cm^2 and the coupling fluid temperature was either 10°C or 37°C. A hydrophone (2.0-mm Needle Hydrophone, Precision Acoustics Ltd., Dorchester, Dorset, UK) was used to monitor the inertial cavitation activity. The hydrophone tip had been coated by the manufacturer with a thin layer of silicone to protect the sensitive piezoelectric element from cavitation damage. At the transducer driving frequency of 20 kHz, the hydrophone had a sensitivity of –236.4 dB$_{re1}$V/µPa. This sensitivity was measured in a calibration by Neptune Sonar Ltd (East Yorkshire, UK). The position of the hydrophone is described in detail in Section 3.3.6 of Robertson and Becker (2018).

The hydrophone was held in place by an aluminum sleeve. The mounting point of the sleeve was fixed relative to the diffusion cell and transducer. Because of the small distance between the transducer and the skin (3 mm) in the present study, it was not possible to position the hydrophone confocally with the transducer. Instead, the hydrophone was focused toward the gap between the transducer tip and the donor chamber. The distance between the tip of the hydrophone and the bottom edge of the transducer was 5 mm. The hydrophone tip was 7 mm from the skin aperture during sonoporation.

The voltage signal recorded by the hydrophone during sonoporation was passed through a pre-amplifier (HP Series Submersible Preamplifier, Precision Acoustics Ltd., Dorchester, Dorset, UK) and a DC coupler (DC Coupler, Precision Acoustics Ltd.). The voltage signal was then read by an oscilloscope (TDS 2014 B, Tektronix, Beaverton, OR, USA) and transferred to a PC running SignalExpress (NI LabVIEW SignalExpress, National Instruments) *via* a USB cable. The oscilloscope collected 2.5-ms duration of data at a sampling rate of 1 MHz every 1 to 2 s.

The hydrophone data were filtered to isolate the passband between 22.5 and 27.5 kHz using MATLAB's “designfilt” function (MATLAB, The MathWorks, Natick, MA, USA) with a filter order of 20. The inertial cavitation dose (ICD) was calculated in a manner similar to that described by Chen et al. (2003) and Hallow et al. (2006), by numerically integrating (in time) the noise amplitude (root mean square of the filtered data for each data set) for the 10 min of continuous ultrasound application in each experiment. Numerical integration was evaluated using the MATLAB “trapz” function.

Transdermal transport experiments

The receiver chambers were filled with room temperature (19 ± 1°C) PBS. A skin sample was then taken from the freezer and visually checked for any holes or areas of non-uniform thickness. Each sample was clamped between the filled receiver chamber and the donor chamber. After ensuring that no air bubbles were present in the receiver chamber, the diffusion cell was mounted into the temperature regulation setup, which involved attaching the silicone tubing to the donor ports and moving the thermocouple into position. De-ionized water at room temperature was then added to the donor chamber and allowed to sit for 3 min. The pump was then switched on so that the coupling fluid circulated through the system. The water bath temperatures required to achieve each of the coupling fluid target temperatures (13°C, 33°C or 46°C) during sonoporation are given in Table 1. For each of the coupling fluid target temperatures, an additional set of experiments were conducted with coupling fluid circulation (but without ultrasound); the water bath temperatures needed for these are also given in Table 1.

Table 1 Water bath temperatures required to achieve the coupling fluid target temperatures for the pre-treatment protocols

alt-text: Table 1

	Protocol					
	Circulation only			Sonoporation at 55 W/cm^2		
Target temperature (°C)	13	33	46	13	33	46
Water bath temperature (°C)	13	35	50	1	25	40

The coupling fluid was circulated for 10 min with continuous ultrasound application at 55 W/cm^2, or without ultrasound in the pure circulation cases. The temperature of the coupling fluid was continuously monitored with the thermocouple positioned outside the transducer beam. After this 10-min period, the coupling fluid was removed from the donor chamber, and the diffusion cell was moved to a holding rack. A 0.5-mL volume of the calcein solution was then applied to the top of the skin so that its entire surface was submerged. This solution was left to diffuse for 5 h at room temperature (19 ± 1°C). During this time, at 1-h intervals, 0.2-mL samples were taken from the receiver chamber with a pipet (Calibra Digital 832 Macro Pipette, Socorex, Ecublens, Switzerland). The sampled volume was then replaced with an identical volume of fresh PBS. This dilution was later accounted for when calculating the concentration of calcein in the receiver chamber. After each study, each diffusion cell was washed with a surfactant cleaner (Jif, Unilever, France) to remove the calcein, which has a tendency to adhere to the polypropylene.

Spectrofluorometry

The calcein concentrations of receiver cell samples were measured with a spectrofluorometer (Fluorolog-3, Horiba Ltd., Kyoto, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 513 nm. These wavelengths are similar to those used to measure calcein in the literature (El Jastimi and Lafleur 1999; Petronilli et al. 1999; Alvarez-Román et al. 2003; Mueller et al. 2004; Morimoto et al. 2005). The software package FluorEssence (FluorEssence Version 3.5, Horiba Ltd.) was used to control the spectrofluorometer and analyze its output. Each 0.2-mL sample was pipetted into a 1.2-mL quartz crystal cuvette (Starna Ltd, Essex, UK). The remaining 1 mL of the cuvette was then filled with PBS. This dilution was accounted for by multiplying the measured spectrofluorometer intensity by six. The emission intensity values measured by the spectrofluorometer were converted to calcein concentrations using a calibration curve that was produced for this study (see Appendix B of Robertson [2018]). Between measurements, the cuvette was cleaned with de-ionized water and dried with Kimwipes (Kimtech, Kimberly-Clarke, Irving, TX, USA) to ensure that all of the calcein was removed.

Statistical analysis

In the comparison of transport data taken at the three temperatures, statistical analysis of the log transformed data was conducted with a one-way analysis of variance using the MATLAB function “anova1.” The *p* values were generated in *post hoc* testing using the MATLAB function “multcomparefunction.” Statistical analyses of the foil pitting data and the ICD were conducted with a permutation test using the MATLAB function “permutationTest.” This permutation test was used to generate the *p* values in the comparison between the transport data of the skin exposed to ultrasound and the skin exposed to pure circulation at the same temperature.

Results

Coupling fluid temperature

The transient coupling fluid temperatures in response to the application of ultrasound at 55 W/cm^2 were considered for the following protocols: Continuous application with circulation, unregulated (continuous application without circulation), at a 10% duty cycle and at a 50% duty cycle (Fig. 3). Unregulated continuous ultrasound application resulted in the coupling fluid temperature exceeding 45°C in just over 4 min. A 50% duty cycle also resulted in the temperature increasing above 40°C. A 10% duty cycle ensured that the temperature remained below 20°C for the 10-min duration, but an equivalent duration of only 1 min of ultrasound was applied.

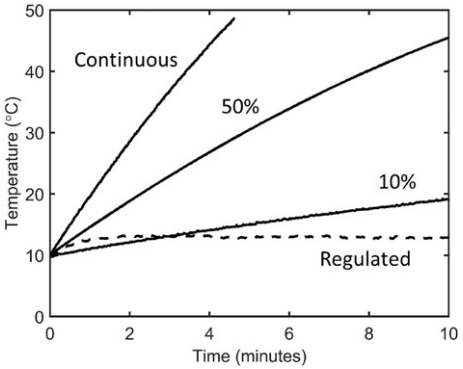

Fig. 3 Coupling fluid temperatures of different application protocols within the modified diffusion cell. Solid lines indicate donor side temperatures with no circulation at continuous application or with 10% or 50% duty cycles. The dashed line represents donor side temperature with circulation undergoing continuous ultrasound application. In all cases, the ultrasound intensity used was 55 W/cm^2.

alt-text: Fig 3

The temperature profiles of the coupling fluid at each of the three target temperatures (13°C, 33°C and 46°C) are illustrated in Figure 4a for both the circulation-only cases and the sonoporation-with-circulation cases. In the cases of ultrasound application, the short initial developmental period (<90 s) reflects the influences of ultrasound heating and circulation cooling as they reach equilibrium to meet the target temperature. After 90 s, the mean and respective 95% confidence interval of the temperatures of the coupling fluid undergoing ultrasound were 12.3°C (standard deviation [SD] = 0.17°C), 33.4°C (SD = 0.30°C) and 46.5°C (SD = 0.22°C).

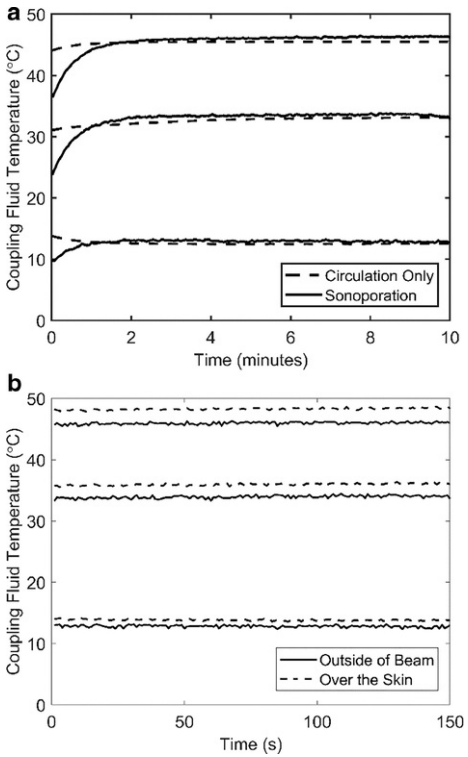

Fig. 4 Coupling fluid temperatures at each target temperature (13°C, 33°C and 46°C). (a) Transient operation of circulation only (dashed line) and sonoporation (solid line). (b) Comparison of temperatures at the skin surface (dashed) and at the position outside of the transducer beam (solid). Continuous ultrasound was applied at an intensity of 55 W/cm^2.

alt-text: Fig 4

In a separate set of experiments, the coupling fluid temperature was measured at two locations simultaneously while ultrasound was applied at an intensity 55 W/cm^2. Here, in addition to the thermocouple placed outside the transducer beam, a thermocouple was placed directly on the skin surface. These temperatures are plotted during a 2.5-min period in which the coupling fluid was thermally fully developed (Fig. 4b). The average differences in the temperatures at these two locations were 0.58°C (SD = 0.25°C), 2.05°C (SD = 0.23°C) and 2.27°C (SD = 0.18) for target temperatures of 13°C, 33°C and 46°C, respectively. In the absence of an applied ultrasound field (when the skin was exposed only to the circulating coupling fluid), there was no noticeable difference in the temperatures of the two locations (data not shown).

Receiver fluid temperature

The receiver fluid temperature 2 mm below the skin was measured for a duration of 30 min. The first 10 min corresponded to the period of coupling fluid circulation (with or without ultrasound). The last 20 min corresponded to the period when the diffusion cell was left at room temperature of 19 ± 1°C (when the donor chamber was emptied of coupling fluid and the calcein solution would be applied to the skin).

In the absence of ultrasound application (Fig. 5a) the receiver fluid experienced sudden temperature increases to 26°C and 32°C when the fluid in the donor chamber was circulated at 33°C and 46°C respectively. When the fluid in the donor chamber was circulated for 10 min at 13°C in the absence of ultrasound, the receiver fluid experienced a very slight decrease in temperature that gradually returned to room temperature thereafter.

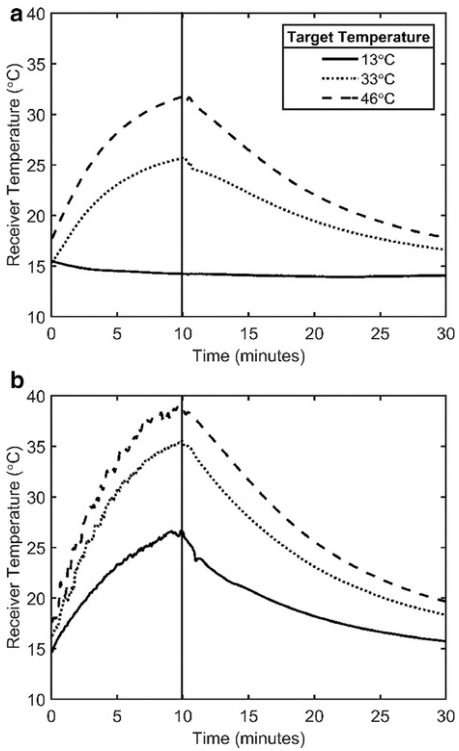

Fig. 5 Receiver chamber temperatures at each of the three target temperatures (13°C, 33°C and 46°C). (a) Pure circulation and (b) circulation with 10 min of continuous ultrasound at an intensity of 55 W/cm^2. The circulation was run for the first 10 min and then turned off (highlighted by the *vertical solid line*). The receiver fluid was then left to return toward room temperature (19 ± 1°C) for the remaining 20 min. Receiver temperatures taken 2 mm below the bottom surface of the skin.

alt-text: Fig 5

The receiver fluid always experienced an increase in temperature during the 10-min ultrasound application, followed by a gradual decrease to room temperature after the coupling fluid was removed (Fig. 5b). The receiver fluid reached maximum temperatures of 27°C, 36°C and 39°C for coupling fluid temperatures of 13°C, 33°C and 46°C, respectively. Note that in all cases illustrated in Figure 5 the initial receiver fluid temperatures near the skin were 1–4°C below room temperature because of heat transfer from the receiver fluid to the frozen skin sample. In the absence of an applied ultrasound field (when the skin was exposed only to the circulating coupling fluid), the difference in the temperatures of the two locations was less than the thermocouple precision of 0.1°C (data not shown).

Permeant solution temperature

After the 10 min of donor fluid circulation (sonoporation or circulation only), the coupling fluid was removed from the donor chamber and the calcein buffer was applied to the skin. The temperature of the calcein-buffer solution was measured for a 15-min period (Fig. 6) after it was applied to the skin. The room temperature during this period was always 19 ± 1°C.

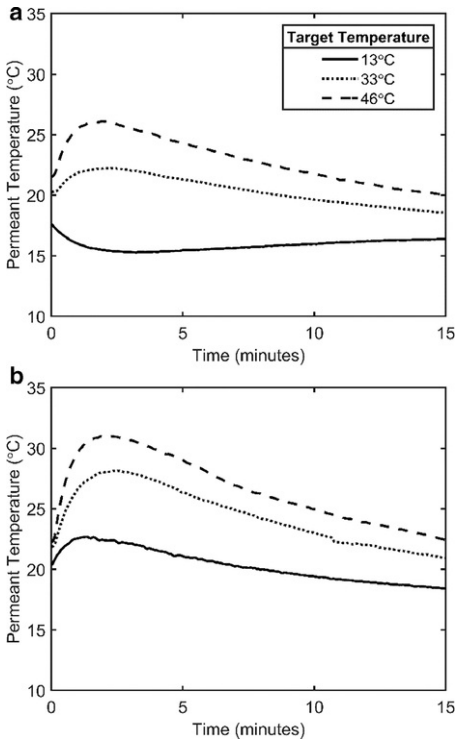

Fig. 6 Permeant temperatures after constant-temperature pre-treatment with (a) pure circulation and (b) circulation with continuous ultrasound at an intensity of 55 W/cm^2. The 15-min period illustrated corresponds to the time immediately after the coupling fluid was replaced witl the calcein-buffer solution.

alt-text: Fig 6

In the absence of ultrasound application, after the skin was exposed to pure circulation at 33°C and 46°C the permeant temperatures experienced slight temperature increases to 22°C and 26°C, respectively, during the first 2.5 min (Fig. 6a). These temperatures gradually tended to room temperature thereafter. After the permeant was applied to skin subjected to pure circulation at 13°C, the permeant temperature dropped to 15°C within 2 min followed by a slow return to room temperature thereafter.

In all cases of skin subjected to ultrasound, the permeant temperatures experienced an initial increase in temperature followed by a gradual decrease tending to room temperature (Fig. 6b). The permeant temperatures peaked within 2.5 min, reaching 23°C, 28°C and 31°C when the coupling fluid was maintained at 13°C, 33°C and 46°C, respectively. At each of the three coupling fluid target temperatures, the permeant temperature was always higher after exposure to ultrasound compared with pure circulation.

Cavitation detection

Foil pitting experiments (n = 10) were used to determine the influence of coupling fluid temperature on the inertial cavitation activity in the present setup. The mean number of pits nearly halved from 15.5 (SD = 9.0) to 12.8 (SD = 5.8), $p = 0.0019$, when the coupling fluid temperature was increased from 10°C to 40°C (Fig. 7a). Decreasing cavitation activity with temperature was also observed in the passive cavitation detection experiments (n = 7). The circulation had no statistical influence on the number of pits, $p = 0.51$ (Fig. 7b). The mean ICD values decreased by one-quarter from 5.09 V-s (SD = 0.26 V-s) to 3.78 V-s (SD = 0.37 V-s), $p < 0.001$, when the coupling fluid temperature was increased from at 10°C to 37°C (Fig. 8). Foil pitting experiments were also used to determine whether the circulation of the coupling fluid during ultrasound application had an influence on the inertial cavitation activity at the skin aperture. In the boxplots of Figures 7–9 the " × " symbol represents the mean values. Any data that exceeded the upper quartile value by more than 1.5 times the interquartile range (represented in Figs. 8 and 9 by the "+" symbol) were still considered when calculating each mean and median.

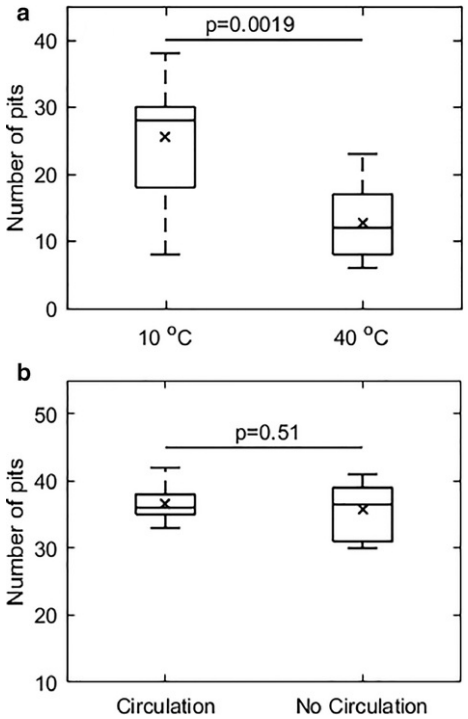

Fig. 7 Number of foil pits. (a) After 1 s of ultrasound application at a coupling fluid temperature of 10°C or 40°C. (b) After 2 s of ultrasound with or without coupling fluid circulation. The ultrasound intensity was 55 W/cm^2 (n = 10).

alt-text: Fig 7

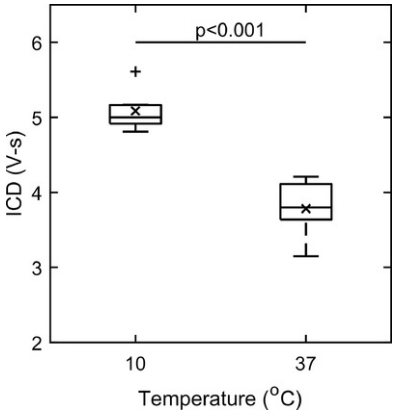

Fig. 8 Influence of coupling fluid temperature on inertial cavitation dose for coupling fluid temperatures of 10°C and 37°C. Ultrasound was applied at an intensity 36 W/cm^2 (n = 7).

alt-text: Fig 8

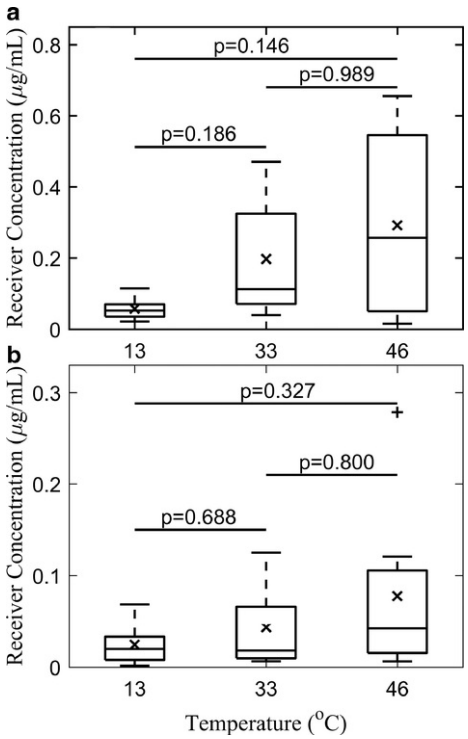

Fig. 9 Receiver chamber calcein concentration 5 h after constant temperature pre-treatment of (a) a 10-min ultrasound application at 55 W/cm^2 (n = 7) or (b) circulation only (n = 7)

alt-text: Fig 9

Transdermal transport

Receiver chamber calcein concentrations were measured at 1-h intervals. Both the mean and the SD of concentration measured 5 h after application increased with coupling fluid temperature. After 5 h (Fig. 9a), the receiver mean calcein concentration of the skin exposed to ultrasound at 13°C was 0.0575 μg/mL (SD = 0.0306 μg/mL). Increasing the coupling fluid temperature to 33°C resulted in an increase in the mean calcein concentration to 0.197 μg/mL (SD = 0.163 μg/mL). At a coupling fluid temperature of 46°C, the mean receiver calcein concentration was 0.292 μg/mL (SD = 0.265 μg/mL). With such dramatic increases in variance with increasing coupling fluid temperature, we found that the transport after ultrasound at 10°C only approached statistically significant difference compared with the other cases, although the significance of the difference was much worse between receiver concentrations after ultrasound was applied to coupling fluid at 46°C than in those after ultrasound was applied at 33°C.

In the absence of ultrasound, increasing the temperature at which the skin was exposed to pure circulation for 10 min resulted in increased mean calcein concentrations, although none of the differences were statistically significant (Fig. 9b). After 5 h, the mean receiver calcein concentration of the skin exposed to circulation at 13°C was 0.0247 μg/mL (SD = 0.0226 μg/mL). The mean concentration of skin exposed to water at 33°C was 0.0435 μg/mL (SD = 0.0440 μg/mL) and that of skin exposed to circulation at 46°C was 0.0774 μg/mL (SD = 0.0967 μg/mL).

The application of ultrasound compared with pure circulation at the same temperature had a statistically significant influence on donor chamber concentrations at 5 h with $p = 0.0239$ at 13°C and with $p = 0.0166$ at 33°C. At 46°C the difference between the receiver concentrations fell outside of the statistically significant range with $p = 0.102$.

Discussion

The modified Franz diffusion cell was able to maintain the average temperature of the coupling fluid to within ±1°C of the target temperatures during continuous ultrasound exposure at an intensity of 55 W/cm^2 (Figs. 3 and 4a). We found that the circulation of the coupling fluid had no significant short-term effect on the cavitation activity in the Franz cell (Fig. 7b), so it is unlikely that the presence of circulation (by itself) had any effect on the sonication of

the skin. The cooling of the coupling fluid by circulation allows the skin to be exposed to continuous ultrasound without the risk of excessive heating, and it should be considered as an alternative to the current methods of duty cycles and/or fluid replacement as it reduces the total time required for the application of ultrasound (by up to 90%).

With the ability to maintain a constant coupling fluid temperature, we were able to explicitly consider the effects of coupling fluid temperature on cavitation activity and subsequent transdermal delivery. Within the modified Franz diffusion donor, increasing the coupling fluid temperature acted to decrease evidence of internal cavitation activity in the forms of foil pitting (Fig. 7a) and of ICD (Fig. 8). This result was anticipated because the threshold pressure required for the onset of cavitation has been reported to decease with temperature in the range 17–35°C (Bader et al. 2012), and the lower pressure is understood to result in a lower jetting velocity (Brennen 1995); it is the jetting that is believed to result in the localized regions of increased permeability within the skin (Polat et al. 2011). However the reduction in the magnitude of the cavitation with temperature did not directly correlate with decreased skin permeability changes; the results of the transport study indicate that transdermal delivery increases with coupling fluid temperature (Fig. 9a). So the question arises: How can transport not decrease with decreased cavitation at higher temperatures?

It is well accepted that temperature has a positive influence on the effective diffusion coefficient of molecular transport through the skin (scaling with temperature as $e^{-1/T}$) (Shahzad et al. 2015). However, it is unlikely that the influence of temperature on diffusion resulted in the increased transport at higher temperatures (Fig. 9a) because measurements of the solute temperature after the ultrasound application (Fig. 6b) reveal that the elevated temperatures of the solute are both minor (<~10°C) and short lived (~15 min) compared with the time scale of transdermal diffusion, which in this study is on the order of hours.

Therefore the increased transport associated with higher coupling fluid temperatures is more likely to be related to the thermally associated weakening of the SC lipid chains in this temperature range (Gay et al. 1994; Clarys et al. 1998; Tang et al. 2002a; Akomeah et al. 2004; Silva et al. 2006). Although we did not directly measure the skin temperature during the transport experiments, in a separate set of experiments the temperatures measured at the skin surface were always within 2°C of the temperatures measured simultaneously at the location outside of the beam (Fig. 7b). Furthermore, the maximum receiver fluid temperatures measured near the skin during the application of ultrasound were always below the coupling fluid target temperatures. It is reasonable to consider that during the transport experiments, the temperature at the skin surface was also close to the measured temperature of the coupling fluid.

Taken together, these results suggest that increasing the skin temperature must have decreased the ability of the SC lipids to withstand the stresses resulting from the inertial cavitation near the skin. This is supported in previous studies in which it has been reported that the SC experiences a dramatic drop in yield strength with temperature (Papir et al. 1975). In a ballistic particle delivery study by Kendall et al. (2004), particle penetration depth into porcine ear SC increased by twofold when the ambient temperature was increased from 20–40°C, reflecting a dramatic drop in the ability of the SC to resist shear stresses in this temperature range. It is reasonable to conclude that even though there was less inertial cavitation at higher coupling fluid temperatures, the weakened SC lipid structure at higher temperatures was more susceptible to the introduction of defects from the jetting of cavitation near the skin.

Clearly, caution should be taken when interpreting the results of Figure 9 in determining the influence of coupling fluid temperature on subsequent transport. The small number of data points taken ($n = 7$) do not result in statistically relevant differences, though it is also very clear that these distributions are not similar at different temperatures. One observation of this study is that the normalized relative SD of the transport after ultrasound nearly doubles as the temperature increases from 13–46°C. Although further research is needed, these observations could not have been made with previous experimental methods.

On a final note, the thermal analysis of the receiver chamber illustrated in Figure 5 provides some insight into controlling anticipated heating of the subcutaneous tissue in *in vivo* applications. Lower coupling fluid temperatures resulted in lower receiver fluid temperatures in the presence of ultrasound (Fig. 5b). Using a circulation to reduce coupling fluid temperatures in clinical applications or *in vivo* could reduce the potential of harmful temperature rises of the underlying tissue.

Conclusions

A Franz diffusion cell was modified with circulation of coupling fluid through the donor chamber to make it possible to maintain the coupling fluid at a constant target temperature (13°C, 33°C or 46°C) during 10 min of continuous sonoporation of porcine skin at an ultrasound intensity of 55 W/cm^2. This system could be used to streamline the experimental procedure by removing the need for duty cycles of coupling fluid replacement. Because the coupling fluid temperature was controllable, the system was able to be used to investigate the influence of coupling fluid temperature on cavitation and transport. Increasing coupling fluid temperature resulted in decreased cavitation activity, increased mean transport and increased variance in transport data.

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